A Role for NK Cells as Regulators of CD4$^+$ T Cells in a Transfer Model of Colitis

Madeline M. Fort, Michael W. Leach and Donna M. Rennick

J Immunol 1998;161;3256-3261

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

A Role for NK Cells as Regulators of CD4$^+$ T Cells in a Transfer Model of Colitis[1]

Madeline M. Fort,[2]* Michael W. Leach,† and Donna M. Rennick*

Previous studies have shown that the chronic inflammation observed in the colon of IL-10-deficient (IL-10$^{-/-}$) mice is mediated by CD4$^+$ Th1 T cells and is dependent on the presence of IFN-γ for its initial development. As CD4$^+$ T cells from IL-10$^{-/-}$ mice will cause colitis when transferred into recombinase-activating gene (Rag)-deficient recipients, we considered the possibility that the recipients' NK cells could be an important source of IFN-γ for the development of colitis. Therefore, the ability of IL-10$^{-/-}$ CD4$^+$ T cells to cause colitis in Rag-deficient recipients that had been depleted of NK cells was tested. Contrary to our expectations, NK cell-depleted recipients of IL-10$^{-/-}$ CD4$^+$ T cells developed accelerated disease compared with nondepleted recipients. Furthermore, CD4$^+$ T cells from normal mice (IL-10$^{+/+}$) also caused colitis in NK cell-depleted recipient mice, but not in nondepleted recipients. NK cells inhibited effector CD4$^+$CD45RB^{high} T cells, and subsequent experiments showed that this effect was dependent on perforin. Thus NK cells can play an important role in down-regulating Th1-mediated colitis by controlling the responses of effector T cells to gut bacteria. *The Journal of Immunology,* **1998, 161: 3256–3261.**

Inflammatory bowel disease (IBD)[3] in humans is a debilitating condition that presents as chronic inflammation affecting predominantly the large intestine, as in ulcerative colitis, or discontinuous portions of the entire gastrointestinal tract, as in Crohn's disease (1–4). Studies of immune-reactive cells and proinflammatory mediators in the intestinal tissue of ulcerative colitis or Crohn's disease patients has suggested that IBD is the result of dysregulated immune responses to enteric Ags (5–7). Several different mouse models of spontaneous IBD have been characterized, including IL-10-deficient (IL-10$^{-/-}$), TGF-β-deficient, IL-2-deficient, TCRα-chain-deficient, and Gαi-deficient mice (8–13). Additional murine models of chronic intestinal disease have been generated by disrupting the balance between pro- and anti-inflammatory elements either by chemical insult (14) or by the reconstitution of immunodeficient mice with subsets of CD4$^+$ T cells (15–17). All of these models are being studied in an effort to identify the pathogenic mechanisms responsible for initiating and/or sustaining human IBD.

Th1 cells are primary mediators of intestinal disease in the majority of these IBD models (10, 14, 18, 19). Although it is well established that Th1 T cells provide protection against bacterial and parasitic infections (reviewed in Refs. 20 and 21), there is now evidence that the uncontrolled generation and/or activation of Th1 cells may underlie the immunopathologic changes seen in a variety of models of chronic inflammation (i.e., colitis, thyroiditis, pancreatitis, nephritis, and experimental allergic encephalitis). Given the potential of Th1 cells to play either a protective or pathogenic role, we have previously investigated the role of IFN-γ in the development and maintenance of the colitis seen in IL-10$^{-/-}$ mice. Treatment of neonatal IL-10$^{-/-}$ mice with anti-IFN-γ mAb dramatically delayed the onset and reduced the severity of colonic inflammation. However, anti-IFN-γ mAb treatment of IL-10$^{-/-}$ adults had no effect on their established disease (19, 22, 23). Therefore, we concluded that IFN-γ is a critical component required for initiating, but not for sustaining, CD4$^+$ Th1-dependent colitis in IL-10$^{-/-}$ mice. This conclusion is consistent with the ability of anti-IFN-γ mAb treatment to prevent the development of colitis in a CD4$^+$ T cell transfer model (24).

Given the importance of IFN-γ during the inductive phase of a pathogenic Th1 response in IL-10$^{-/-}$ mice, we questioned whether NK cells are a primary source of this early IFN-γ production. In other experimental models, NK cells have been shown to be involved in the differentiation of naive CD4$^+$ T cells into Th1 cells (25). Infectious organisms, such as *Listeria monocytogenes* and *Toxoplasma gondii*, are capable of stimulating macrophages and/or dendritic cells to secrete IL-12 and TNF-α, which in turn induce NK cells to produce IFN-γ (26, 27). This production of IFN-γ by NK cells early in the immune response is critical for the induction of a rapid healing Th1 response to several different infectious organisms, including *Leishmania major*, *T. gondii*, *L. monocytogenes*, and murine cytomegalovirus (28–34). Moreover, in vitro studies have shown that IL-10 is a potent suppresser of IFN-γ production by NK cells because it inhibits the ability of accessory cells to produce the factors necessary for NK cell activation (35, 36). Therefore, as it seemed likely that the dysregulated interactions of NK cells and accessory cells may serve to initiate the development of Th1-mediated colitis in IL-10$^{-/-}$ mice, we tested the possibility that NK cells were a contributing factor to Th1-mediated colitis.

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or from Taconic Farms, (Germantown, NY). Recombinase-activating gene 1-deficient/C57BL/6 (Rag1$^{-/-}$) mice were either from The

*Department of Immunobiology, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA 94304; and †Schering Plough Research Institute, Schering Plough Corporation, Lafayette, NJ 07848

Received for publication April 6, 1998. Accepted for publication May 27, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] DNAX Research Institute is supported by Schering-Plough Corporation.

[2] Address correspondence and reprint requests to Dr. Madeline Fort, DNAX Research Institute, 901 California Ave., Palo Alto, CA 94306. E-mail address: fort@dnax.org

[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; IL-10$^{-/-}$, IL-10-deficient; pfp$^{-/-}$, pore-forming protein-deficient; Rag1$^{-/-}$ mice, recombinase-activating gene 1-deficient mice; RB^{high}, CD4$^+$CD45RB^{high}-expressing cells; RB^{low}, CD4$^+$CD45RB^{low}-expressing cells; WT, wild-type (IL-10$^{+/+}$); PE, phycoerythrin; B6 mice, C57BL/6 mice; ASGM1, asialo GM$_1$; FasL, Fas ligand; SAS, saturated ammonium sulfate.

0022-1767/98/$02.00

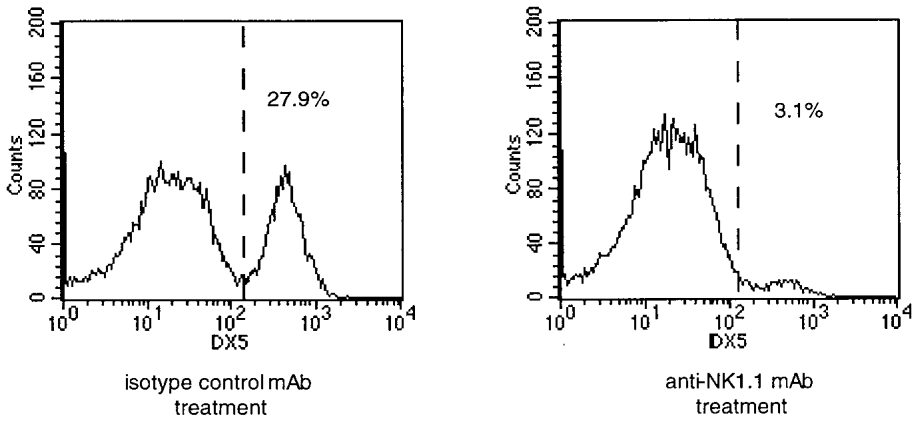

FIGURE 1. Weekly injections of 1 to 2 mg of anti-NK1.1 (PK136) SAS-cut ascites results in the depletion of 80 to 90% of NK cells. Spleens from anti-NK1.1-treated and isotype control-treated Rag1$^{-/-}$ mice were stained with the pan anti-NK cell Ab DX5 and analyzed by flow cytometry. The histograms show the percentage of DX5$^+$ cells within a lymphocyte gate.

Jackson Laboratory or from a colony maintained at the DNAX Animal Care Facility. Pore-forming-protein/Rag2-deficient (pfp$^{-/-}$/Rag2$^{-/-}$) B6/129 outbred mice were obtained from Taconic Farms; B6/129 outbred mice were generated at the DNAX Animal Care Facility. The immunodeficient mice were housed in micro isolator cages in a specific pathogen-free facility and were given only sterile bedding, food, and water. Immunocompetent mice were kept in conventional housing.

Antibodies

Both the PK136 clone (anti-NK1.1) and the L243 clone (anti-human MHC class II; isotype control) were obtained from American Type Culture Collection (Manassas, VA). Ascites was made from each of these clones at Harlan Bioproducts for Science (Madison, WI) and tested for endotoxin. Mice were given 2 mg of Ig from saturated ammonium sulfate (SAS) cut PK136 i.p., followed by weekly i.p. injections of 1 mg. The isotype control mice were given 0.5 to 0.8 mg of SAS cut L243 i.p. at all time points. The anti-asialo-GM$_1$ Ab (Wako Chemicals, Richmond, VA) was reconstituted according to the manufacturer's specifications, and 50 μl was given i.p. on a weekly basis. All depleting or control Abs were given to the mice the day before cell transfer, and then every 6 to 8 days for the duration of the experiment. Efficacy of the in vivo depleting Ab was determined by flow cytometric analysis of the spleens of treated mice. After RBC lysis, spleen cells of treated mice were stained with anti-CD4 FITC (Caltag, Burlingame, CA), anti-NK1.1 PE (PharMingen, San Diego, CA) and, as a separate NK cell marker, biotinylated DX5 (PharMingen), followed by streptavidin-PE (Caltag), and analyzed by flow cytometry (see Fig. 1).

Cell isolations and transfers

CD4$^+$ splenic T cells were obtained by two separate methods, depending on the experiment. In the first method (see Tables I and II; Fig. 2), spleens were first enriched for CD4$^+$ T cells by red cell lysis and magnetic bead depletion using lineage-specific mAb supernatants (10% v/v): B220 (B cells) and Ter119 (erythrocytes). mAb-stained cells were removed in a magnetic field using goat anti-rat IgG (Fc) and goat anti-rat IgG (H + L)-coated magnetic beads (PerSeptive Diagnostics, Cambridge, MA). Remaining cells were then stained with anti-CD4-FITC and anti-NK1.1 PE or with anti-CD4-PE (Caltag) and anti-CD45RB-FITC (PharMingen) for cell sorting. Two-color cell sorting was performed using a FACStar^{Plus} (Becton Dickinson, Mountain View, CA); the sorted CD4$^+$NK1.1$^-$, CD4$^+$CD45RB^{low}, and CD4$^+$CD45RB^{high} T cells were >98% pure upon reanalysis. Alternatively (see Fig. 4), splenic cells were stained after RBC lysis successively with anti-CD4 biotin (PharMingen), streptavidin-FITC (Biomedia, Foster City, CA), and magnetic cell separation system (MACS) biotin microbeads (Miltenyi Biotec, Auburn, CA). The cells were then run over successive MACS VS$^+$ columns until a purity of >95% was obtained by flow cytometric analysis. Purified CD4$^+$ cells (1×10^5) were injected i.p. into Rag1$^{-/-}$ or pfp$^{-/-}$/Rag2$^{-/-}$ recipient mice. From 4 to 6 wk after T cell transfer, mice were sacrificed and analyzed for the presence of enterocolitis.

Analysis of IBD

Microscopic examination of mouse large intestine was performed in a blinded fashion by the same pathologist (M. W. Leach) on formalin-fixed tissue sections stained with hematoxylin and eosin, as previously described (19). As the lesions in IL-10$^{-/-}$ mice can be multifocal, longitudinal sections of the entire length of the colon were evaluated, taking into account both the number of lesions and their severity. Each region of the colon (cecum; ascending, transverse, and descending colon; and rectum) was graded semiquantitatively as 0 (no change) to 5 (most severe change). The grading represents an increasing incidence and severity of transmural inflammation, goblet cell loss, crypt abscesses and ulceration, and fibrosis in the lamina propria. The summation of the score for each segment of the colon provides a total disease score per mouse (0–25) where: 0 indicates no change; 1 to 5, mild disease; 6 to 10, mild-moderate; and 11 to 20, severe. No mice in these studies had a score above 20 because such severe disease results in death.

Results and Discussion

IL-10$^{-/-}$ CD4$^+$ T cells expand more rapidly and cause more severe disease in NK cell-depleted Rag1$^{-/-}$ recipients

To determine if early IFN-γ production by NK cells was important for the development of the pathogenic CD4$^+$ T cells, we used a CD4$^+$ T cell transfer model. We have previously shown that purified CD4$^+$ T cells from IL-10$^{-/-}$ mice will cause colitis when transferred into immunodeficient mice (8, 37). To determine if the colitis that develops in immunodeficient recipients depended on the presence of IFN-γ-producing host NK cells, we transferred IL-10$^{-/-}$ CD4$^+$ T cells into NK cell-depleted immunodeficient mice. NK cell-depleted recipient mice were created by treating Rag1$^{-/-}$ (B6) mice with the anti-NK1.1 mAb PK136 in vivo. The ability of PK136 to deplete NK cells in B6 mice has been well documented (38). Flow cytometric analysis of spleens from anti-NK1.1-treated Rag1$^{-/-}$ mice showed at least 80 to 90% depletion of NK cells (Fig. 1).

Purified splenic CD4$^+$ T cells from IL-10$^{-/-}$ mice were injected into anti-NK1.1-treated or isotype control-treated Rag1$^{-/-}$ mice. Importantly, the IL-10$^{-/-}$ CD4$^+$ T cells that were transferred did not contain the rare NK1.1$^+$ CD4$^+$ T cell subset (see *Materials and Methods*) and, thus, any differences seen in anti-NK1.1-treated recipients were due to the elimination of NK cells rather than NK1.1$^+$ T cells. Results from previous experiments have shown that colitis develops in Rag2$^{-/-}$ recipients 6 to 8 wk after the transfer of IL-10$^{-/-}$ CD4$^+$ T cells (8, 37). In this case, however, the experiment was terminated early because the NK cell-depleted Rag1$^{-/-}$ recipients were moribund by 4 wk after cell transfer. Histological analysis confirmed our gross observations that the NK cell-depleted recipients of IL-10$^{-/-}$ CD4$^+$ T cells had developed moderate to severe colitis, whereas only mild colitis was found in the isotype control-treated recipients at this time point (Table I). The colitis was characterized by epithelial

Table I. *NK cell-depleted Rag1$^{-/-}$ recipients of IL-10$^{-/-}$ CD4$^+$ T cells develop a more accelerated colitis*

Donor Lymphocytes[a]	In Vivo Ab Treatment	Incidence	Colitis Score[b]
IL-10$^{-/-}$ CD4$^+$NK1.1$^-$	Isotype control	4/5	2.2 ± 1.0
IL-10$^{-/-}$ CD4$^+$NK1.1$^-$	Anti-NK1.1	5/5	11.8 ± 3.6[c]

[a] A total of 1×10^5 purified splenic CD4$^+$NK1.1$^-$ cells were transferred i.p. into Rag1$^{-/-}$ mice that were either treated with either anti-NK1.1 mAb or isotype control mAb. See *Materials and Methods* for in vivo Ab treatments.

[b] Mean colitis score ± SD of affected mice determined 4 wk after transfer as described in *Materials and Methods*.

[c] Significantly different from isotype control mice; Mann-Whitney nonparametric test; $p < 0.02$.

hyperplasia, which resulted in crypt hypertrophy, mucin depletion from goblet cells, and inflammatory cell infiltrates, which consisted of mononuclear cells accompanied by smaller numbers of neutrophils and rare eosinophils (data not shown, but see below). Furthermore, increased disease severity correlated with a higher number of donor αβTCR$^+$ CD4$^+$ T cells found by flow cytometric analysis of the spleens of anti-NK1.1-treated (average of 1.5×10^6 CD4$^+$ T cells/spleen) vs isotype control-treated recipients (average of 0.6×10^6 CD4$^+$ T cells/spleen). Therefore, our findings suggested that the absence of NK cells actually augmented the ability of IL-10$^{-/-}$ CD4$^+$ T cells to cause inflammation in the colon.

Wild-type (WT) CD4$^+$ T cells cause colitis in NK cell-depleted Rag1$^{-/-}$ recipients

Our previous studies with IL-10$^{-/-}$ mice have shown that they develop colitis because an important regulatory subpopulation of CD4$^+$ T cells is either absent or dysfunctional due to their inability to produce IL-10 (22, 37). Hence the inhibitory effect of NK cells on the development of colitis may be detectable only during the transfer of an IL-10-deficient CD4$^+$ T cell population. This implies that a WT, IL-10-producing, CD4$^+$ T cell population would not cause colitis in NK cell-deficient recipient mice. To test this, we transferred purified splenic CD4$^+$ T cells from WT C57BL/6 mice into anti-NK1.1-treated or into isotype control-treated Rag1$^{-/-}$ recipients. As shown in Figure 2, 80% of anti-NK1.1-treated Rag1$^{-/-}$ recipients of WT CD4$^+$ T cells developed colitis within 4 to 6 wk after transfer. The colitis in the anti-NK1.1-treated recipients of WT CD4$^+$ T cells (Fig. 3*D*) was morphologically similar to that seen in anti-NK1.1-treated recipients of IL-10$^{-/-}$ CD4$^+$ T cells. The transfer of low numbers of CD4$^+$ T cells into immunodeficient mice has been shown by others to result in the development of colitis 3 to 6 mo after the T cell transfer (17, 39, 40). In our studies, however, WT CD4$^+$ T cells only rarely induced colitis in isotype control-treated Rag1$^{-/-}$ mice 4 to 6 wk after transfer (Figs. 2 and 3*C*). These results indicate that NK cells can play an important regulatory role in the prevention of colitis in immunodeficient recipients irrespective of the source of the transferred CD4$^+$ T cell population.

It was also possible that these results were an artifact of anti-NK1.1 mAb treatment. NK1.1 is a known activating receptor for NK cells, and immobilized PK136 has been shown to trigger IFN-γ production from NK cells in vitro (41). Therefore, anti-NK1.1 mAb treatment in vivo may cause NK cells to release IFN-γ before their depletion. To investigate this possibility, Rag1$^{-/-}$ recipient mice were also depleted of NK cells using anti-asialo GM$_1$ (anti-ASGM1) Ab, which has been shown to deplete NK cells in vivo but does not activate NK cells (38). The transfer of 1×10^5 WT CD4$^+$ T cells into ASGM1-treated Rag1$^{-/-}$ recipients resulted in colitis of equivalent incidence and severity as that seen in anti-NK1.1-treated recipients (Fig. 2). Importantly, NK cell-depleted Rag1$^{-/-}$ mice that did not receive T cells did not develop colitis and remained healthy throughout the experimental time period (Figs. 2 and 3). This control shows that NK cell depletion in and of itself does not result in colonic inflammation in immunodeficient mice.

CD4$^+$CD45RB^{low} regulatory T cells engraft normally in NK cell-depleted Rag1$^{-/-}$ mice

The transfer of WT CD4$^+$ T cells into syngeneic Rag-deficient or SCID mice generally leads to reconstitution without signs of a pathogenic response in the gastrointestinal tract (15, 16). Recently, it was demonstrated that this outcome is dependent on the simultaneous engraftment of effector and regulatory CD4$^+$ T cells, which express high and low levels of the CD45RB molecule, respectively. Purified effector CD4$^+$CD45RB^{high} (RB^{high}) T cells caused colitis upon transfer into immunodeficient recipients unless cotransferred with CD4$^+$CD45RB^{low} (RB^{low}) T cells, which are enriched for regulatory T cells (15, 16, 18). Our finding that unseparated WT CD4$^+$ T cells caused an unusually high incidence of colitis in NK cell-depleted Rag1$^{-/-}$ recipients suggested that NK

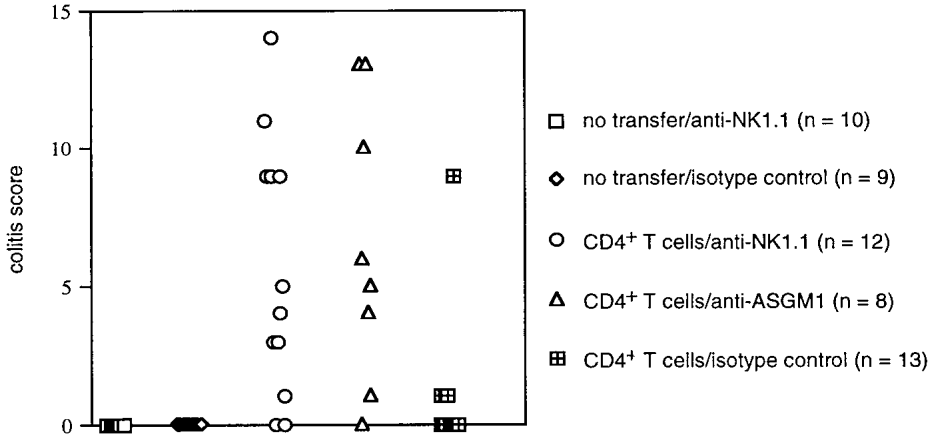

FIGURE 2. IL-10$^{+/+}$ CD4$^+$ T cells cause colitis in NK cell-depleted Rag1$^{-/-}$ mice. Recipient mice were treated with either anti-NK1.1, anti-ASGM1, or isotype control Ab and then either not transplanted (no transfer) or transplanted with 1×10^5 purified CD4$^+$ (NK1.1$^-$) B6 splenocytes 1 to 2 days later. Mice were sacrificed 4 to 6 wk after T cell transfer. Each symbol represents an individual mouse and data is pooled from three separate experiments.

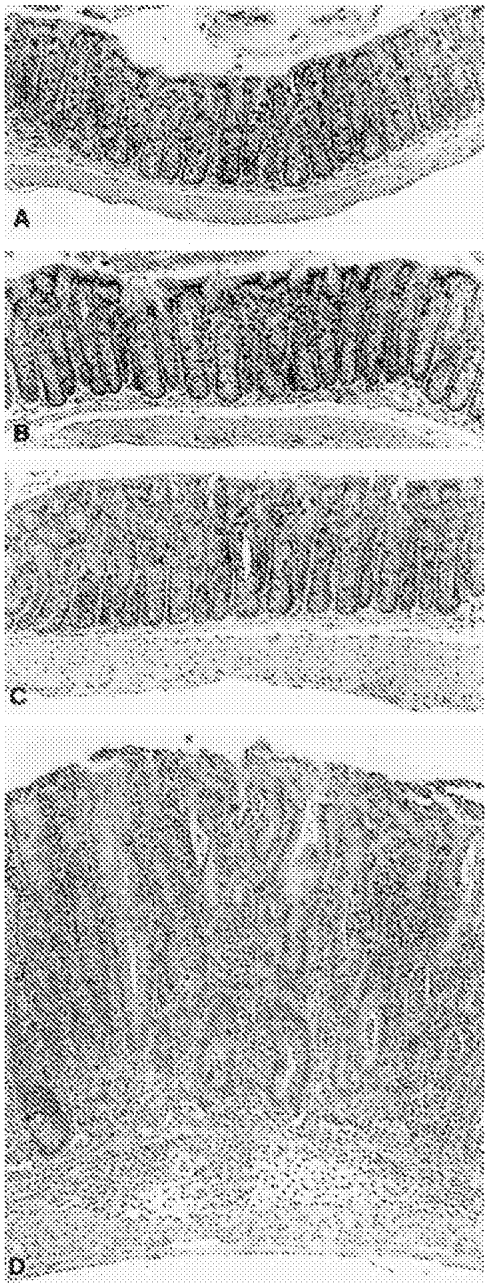

FIGURE 3. Histopathologic analysis of colons from Rag1$^{-/-}$ mice (×95, H&E stain). The lumen of the colon is at the top of each photomicrograph. Representative distal colons from Rag1$^{-/-}$ mice receiving isotype control mAb only (*A*), anti-NK1.1 mAb only (*B*), or WT CD4$^+$ T cells and isotype control mAb (*C*). There are no microscopic changes present in these three groups. Representative distal colon from a mouse receiving WT CD4$^+$ T cells and anti-NK1.1 mAb (*D*), demonstrating epithelial hyperplasia, crypt hypertrophy with slight irregularity of the glands, and inflammatory cell infiltrates in the mucosa and submucosa. A few crypt abscesses are present as well. The epithelial hypertrophy and mucosal inflammation have resulted in thickening of the mucosa.

cells were somehow necessary to insure a balanced reconstitution by effector and regulatory CD4$^+$ T cell populations.

We hypothesized that NK cells could prevent colitis in recipients of WT CD4$^+$ T cells either by inhibiting the generation of effector RB^{high} T cells or by aiding in the establishment of regulatory RB^{low} T cells. To determine if NK cells were necessary for the establishment of regulatory T cells, WT RB^{low} T cells were transferred into NK cell-depleted or NK cell-containing Rag1$^{-/-}$ mice. These same recipients were then transplanted with RB^{high} T cells 21 days later. By allowing the RB^{low} T cells 3 wk to expand and home to the relevant tissues, we hoped to create recipient mice that had an established regulatory T cell population before transferring in the effector RB^{high} T cells. Treatment with anti-NK1.1 or isotype control mAb was initiated either at day −1 (i.e., before the RB^{low} T cell transfer) or at day 20 (i.e., before the RB^{high} T cell transfer). Recipient mice were sacrificed 6 wk after the RB^{high} T cell transfer (see Table II).

In agreement with previously published data (15, 16, 18), transfer of only RB^{high} cells into either isotype control-treated or anti-NK1.1-treated Rag1$^{-/-}$ recipients resulted in the majority of mice developing moderate to severe colitis, while control-treated recipients of both RB^{low} and RB^{high} cells either did not develop colitis or had mild inflammation (Table II, lines 1 and 2). When recipients of RB^{low} T cells that were depleted of NK cells just prior to receiving RB^{high} T cells, four out of five did not develop colitis (Table II, line 3). This result suggests that the presence of NK cells is unnecessary for the ability of an established regulatory T cell population to control effector T cells. Importantly, RB^{low} cells that had been transferred directly into anti-NK1.1-treated recipients were capable of preventing colitis by the RB^{high} subset (Table II, line 4). These results show that NK cells are not necessary for the establishment of functional RB^{low} T cells and, thus, suggest that NK cells must be affecting RB^{high} T cells. Therefore, we conclude that when unfractionated CD4$^+$ T cells are transferred into immunodeficient recipients, in which no established regulatory T cell population exists, NK cells are required for the control of effector T cell responses to enteric bacteria.

The inhibitory role of NK cells in colitis is perforin dependent

The ability of NK cells to inhibit effector T cells is contrary to the characterization of NK cells as promoters of Th1-mediated inflammatory responses. However, our data are supported by increasing evidence that NK cells may play a regulatory role in some Th1-mediated immune responses, such as pneumonitis, autoimmunity in *lpr* mice, and experimental allergic encephalitis in B6 mice (42–44). The mechanisms for this inhibitory effect of NK cells on Th1-mediated responses are currently unknown. Activated NK cells can secrete TGF-β (45), and TGF-β has been implicated in the prevention of colitis in several models (12, 13, 46). NK cells also secrete TNF-α, which can have cytotoxic effects on activated T cells (47). However, NK cells are best characterized by their ability to lyse target cells in a perforin-dependent manner. NK cells from mice that are deficient in perforin (pfp$^{-/-}$ mice) are unable to lyse allogeneic, virally infected, or NK cell-sensitive targets (48). Therefore, we considered the possibility that NK cells might be lysing effector T cells by a perforin-dependent mechanism to prevent the induction of colitis in our T cell transfer system.

To test the role of perforin in our model of colitis, we transferred CD4$^+$ T cells into immunodeficient mice that were also deficient in perforin (pfp$^{-/-}$/Rag2$^{-/-}$ mice). The pfp$^{-/-}$/Rag2$^{-/-}$ mice contain no T or B cells and their NK cells are unable to lyse targets by a perforin-mediated mechanism. As a positive control, some recipients were physically depleted of NK cells by anti-ASGM1 Ab treatment prior to T cell transfer. As shown in Figure 4, 67% of NK cell-depleted pfp$^{-/-}$/Rag$^{-/-}$ recipients developed mild to moderate colitis by 4 wk after T cell transfer. The mild colitis observed in these pfp$^{-/-}$/Rag2$^{-/-}$ recipients as opposed to Rag1$^{-/-}$ recipients (Fig. 4 vs Fig. 2) is most likely due to the differences in genetic background (see *Materials and Methods*) (19). In comparison, pfp$^{-/-}$/Rag2$^{-/-}$ recipients of CD4$^+$ T cells developed colitis with the same incidence and severity as in their NK cell-depleted counterparts (Fig. 4). As there was no significant

Table II. *The absence of NK cells does not affect the establishment or regulatory function of CD4$^+$CD45RB^{low} T cells*

mAb (Day −1)[a]	Cells transferred[b] (Day 0)	mAb (Day 20)[a]	Cells transferred[b] (Day 21)	Colitis[c] None	Mild	Moderate	Severe
Anti-NK1.1 or control	None		RB^{high}	1	1	4	3
Control	RB^{low}		RB^{high}	5	3		
	RB^{low}	Anti-NK1.1	RB^{high}	4		1	
Anti-NK1.1	RB^{low}		RB^{high}	9	1		

[a] In vivo Ab treatments (anti-NK1.1 or isotype control) of recipient Rag1$^{-/-}$ mice were started on day −1 or day 20 and were continued for the duration of the experiment.
[b] A total of 2.5 × 10^4 purified splenic CD4$^+$CD45RB^{low} (RB^{low}) and/or 5 × 10^4 purified splenic CD4$^+$CD45RB^{high} (RB^{high}) T cells were injected into Rag1$^{-/-}$ recipient mice on either day 0 or day 21.
[c] The presence of colitis in each mouse was assessed 6 wk after day 21. The severity of the colitis was determined as described in *Materials and Methods*. The number of affected and unaffected (no colitis) mice are shown from two separate experiments.

difference between the two groups, we concluded that NK cells use perforin to regulate the development of colitis in our CD4$^+$ T cell transfer model. However, NK cells have also been shown to express Fas ligand (FasL) and to lyse Fas-expressing target cells (49). Therefore, it is possible that perforin is not the only mechanism by which NK cells can inhibit effect on CD4$^+$ T cells, and it will be interesting to determine if Fas/FasL, as well as TGF-β, contribute to the ability of NK cells to inhibit effector T cells.

In summary, we have demonstrated a previously unappreciated role for NK cells as regulators, rather than promoters, of Th1-mediated colitis in a CD4$^+$ T cell transfer system. Furthermore, we have been able to characterize this regulatory effect as being directed against the effector CD4$^+$CD45RB^{high} T cell subset and dependent on perforin. What remains unclear is whether NK cells inhibit effector T cells directly or indirectly. It is possible that NK cells directly lyse activated T cells. Alternatively, it is possible that NK cells control effector T cell activation by eliminating professional APCs, as studies by others have shown that NK cells can lyse B7-1$^+$ macrophages and dendritic cells (50, 51). We are currently performing experiments to differentiate between these two possibilities. Finally, our findings indicate that there is a critical role of NK cells in controlling effector T cell responses. This role is most evident in the absence of an established regulatory CD4$^+$ T cell population. As NK cells are found in the lamina propria of the gut (52) (data not shown), their regulatory effect may be crucial in controlling the extent of local mucosal Th1 cell responses to enteric Ags during the development of regulatory T cell responses.

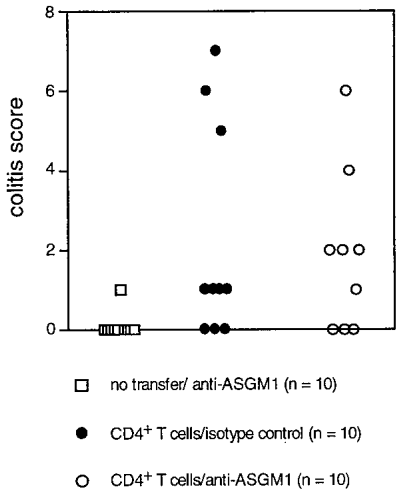

FIGURE 4. Perforin-deficient Rag2$^{-/-}$ recipients of CD4$^+$ T cells develop colitis. Purified splenic CD4$^+$NK1.1$^-$ cells (1 × 10^5) were transferred i.p. into pfp$^{-/-}$/Rag1$^{-/-}$ (129/B6 outbred) hosts that were either depleted of NK cells (anti-ASGM1) or given control Ab. There is no significant difference between the groups that received CD4$^+$ T cells (Mann-Whitney nonparametric test). Data are combined from two separate experiments.

Acknowledgment

We thank Ms. E. Callas, Ms. M. Cook, Ms. D. Polakoff, and Dr. J. Cupp for cell sorting; the Schering-Plough Research Institute Histology Laboratory for technical assistance; and Drs. N. Davidson and L. Lanier for critical reading of the manuscript.

References

1. MacDermott, R. P., and W. F. Stenson. 1988. Alterations in the immune system in ulcerative colitis and Crohn's disease. *Adv. Immunol. 42:285.*
2. Braegger, C. P., and T. T. MacDonald. 1994. Immune mechanisms in chronic inflammatory bowel disease. *Ann. Allergy 72:135.*
3. Podolsky, D. K. 1991. Inflammatory bowel disease (Part I). *N. Engl. J. Med. 325:928.*
4. Podolsky, D. K. 1991. Inflammatory bowel disease (Part II). *N. Engl. J. Med. 325:1008.*
5. Chadwick, V. S., and R. P. Anderson. 1992. Microorganisms and their products in inflammatory bowel disease. In *Inflammatory Bowel Disease.* R. P. McDermott, and W. F. Stenson, eds. Elsevier, New York, p. 241.
6. Sartor, R. B. 1995. Current concepts of the etiology and pathogenesis of ulcerative colitis and Crohn's disease. *Gastroenterol. Clin. North Am. 24:475.*
7. Sartor, R. B. 1997. Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. *Am. J. Gastroenterol. 92:5.*
8. Kühn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Müller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell 75:263.*
9. MacDonald, T. T. 1994. Inflammatory bowel disease in knockout mice. *Curr. Biol. 4:261.*
10. Mizoguchi, A., E. Mizoguchi, C. Chiba, B. M. Spiekermann, S. Tonegawa, C. Nagler-Anderson, and A. K. Bhan. 1996. Cytokine imbalance and autoantibody production in T cell receptor-α mutant mice with inflammatory bowel disease. *J. Exp. Med. 183:847.*
11. Hornquist, C. E., X. Lu, P. M. Rogers-Fani, U. Rudolph, S. Shappel, L. Birnbaumer, and G. R. Harriman. 1997. Gαi_2-deficient mice with colitis exhibit a local increase in memory CD4$^+$ T cells and proinflammatory Th1-type cytokines. *J. Immunol. 158:1068.*
12. Powrie, F. 1995. T cells in inflammatory bowel disease: protective and pathogenic roles. *Immunity 3:171.*
13. Shull, M. M., I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, N. Annunziata, and T. Doetschman. 1992. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. *Nature 359:693.*
14. Neurath, M. F., I. Fuss, B. L. Kelsall, E. Stuber, and W. Strober. 1995. Antibodies to IL-12 abrogate established experimental colitis in mice. *J. Exp. Med. 182: 1280.*
15. Powrie, F., M. W. Leach, S. Mauze, L. B. Caddle, and R. L. Coffman. 1993. Phenotypically distinct subsets of CD4$^+$ T cells induce or protect from chronic intestinal inflammation in C.B-17 scid mice. *Int. Immunol. 5:1461.*
16. Morrissey, P. J., K. Charrier, S. Braddy, D. Liggitt, and J. D. Watson. 1993. CD4 T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice: disease development is prevented by cotransfer of purified CD4$^+$ T cells. *J. Exp. Med. 178:237.*
17. Claesson, M. H., A. Rudolphi, S. Kofoed, S. S. Poulsen, and J. Reimann. 1996. CD4$^+$ T lymphocytes injected into severe combined immunodeficient (SCID) mice lead to an inflammatory and lethal bowel disease. *Clin. Exp. Immunol. 104:491.*
18. Powrie, F., R. Correa-Oliveira, S. Mauze, and R. L. Coffman. 1994. Regulatory interactions between CD45RB^{high} and CD45RB^{low} CD4$^+$ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J. Exp. Med. 179:589.*

19. Berg, D. J., N. Davidson, R. Kühn, W. Müller, S. Menon, G. Holland, L. Thompson-Snipes, M. W. Leach, and D. Rennick. 1996. Enterocolitis and colon cancer in interleukin 10-deficient mice are associated with aberrant cytokine production and $CD4^+$ Th1-like responses. *J. Clin. Invest. 98:1010.*
20. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature 383:787.*
21. Sher, A., and R. L. Coffman. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annu. Rev. Immunol. 10:385.*
22. Davidson, N. J., M. M. Fort, W. Müller, M. W. Leach, and D. M. Rennick. 1998. Chronic colitis in IL-$10^{-/-}$ mice: insufficient counter regulation of a Th1 response. *Int. Rev. Immunol. In press.*
23. Davidson, N. J., S. A. Hudak, R. E. Lesley, S. Manon, M. W. Leach, and D. M. Rennick. IL-12, but not IFN-γ, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J. Immunol. 161:3143.*
24. Powrie, F., M. W. Leach, S. Mauze, S. Menon, L. B. Caddle, and R. L. Coffman. 1994. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with $CD45RB^{hi}$ $CD4^+$ T cells. *Immunity 1:553.*
25. Romagnani, S. 1992. Induction of Th1 and Th2 responses: a key role for the "natural" immune response? *Immunol. Today 13:379.*
26. Tripp, C. S., S. F. Wolf, and E. R. Unanue. 1993. Interleukin 12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA 90:3725.*
27. Gazzinelli, R. T., M. Wysocka, S. Hayashi, E. Y. Denkers, S. Hieny, P. Caspar, G. Trinchieri, and A. Sher. 1994. Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii. J. Immunol. 153:2533.*
28. Scharton, T. M., and P. Scott. 1993. Natural killer cells are a source of interferon γ that drives differentiation of $CD4^+$ T cell subsets and induces early resistance to *Leishmania major* in mice. *J. Exp. Med. 178:1045.*
29. Scharton-Kersten, T., and P. Scott. 1995. The role of the innate immune response in Th1 cell development following *Leishmania major* infection. *J. Leukocyte Biol. 57:515.*
30. Orange, J. S., B. Wang, C. Terhorst, and C. A. Biron. 1995. Requirement for natural killer cell-produced interferon γ in defense against murine cytomegalovirus infection and enhancement of this pathway by interleukin 12 administration. *J. Exp. Med. 182:1045.*
31. Denkers, E. Y., R. T. Gazzinelli, D. Martin, and A. Sher. 1993. Emergence of $NK1.1^+$ cells as effectors of IFN-γ dependent immunity to *Toxoplasma gondii* in MHC class I-deficient mice. *J. Exp. Med. 178:1465.*
32. Sher, A., I. P. Oswald, S. Hieny, and R. T. Gazzinelli. 1993. *Toxoplasma gondii* induces a T-independent IFN-γ response in natural killer cells that requires both adherent accessory cells and TNF-α. *J. Immunol. 150:3982.*
33. Bancroft, G. J., R. D. Schreiber, and E. R. Unanue. 1991. Natural immunity: a T-cell-independent pathway of macrophage activation, defined in the scid mouse. *Immunol. Rev. 124:5.*
34. Dunn, P. L., and R. J. North. 1991. Early γ interferon production by natural killer cells is important in defense against murine listeriosis. *Infect. Immun. 59:2892.*
35. Hsu, D-H., K. W. Moore, and H. Spits. 1992. Differential effects of IL-4 and IL-10 on IL-2-induced IFN-γ synthesis and lymphokine-activated killer activity. *Int. Immunol. 4:563.*
36. Moore, K. W., A. O'Garra, R. de Waal Malefyt, P. Vieira, and T. R. Mosmann. 1993. Interleukin-10. *Annu. Rev. Immunol. 11:165.*
37. Davidson, N. J., M. W. Leach, M. M. Fort, L. Thompson-Snipes, R. Kühn, W. Müller, D. J. Berg, and D. M. Rennick. 1996. T helper cell 1-type $CD4^+$ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. *J. Exp. Med. 184:241.*
38. Ehl, S., R. Nuesch, T. Tanaka, M. Myasaka, H. Hengartner, and R. Zinkernagel. 1996. A comparison of efficacy and specificity of three NK depleting antibodies. *J. Immunol. Methods 199:149.*
39. Bonhagen, K., S. Thoma, P. Bland, S. Bregenholt, A. Rudolphi, H. Claesson, and J. Reimann. 1996. Cytotoxic reactivity of gut lamina propria $CD4^+$ $\alpha\beta$ T cells in SCID mice with colitis. *Eur. J. Immunol. 26:3074.*
40. Rudolphi, A., K. Bonhagen, and J. Reimann. 1996. Polyclonal expansion of adoptively transferred $CD4^+$ $\alpha\beta$ T cells in the colonic lamina propria of scid mice with colitis. *Eur. J. Immunol. 26:1156.*
41. Arase, H., N. Arase, and T. Saito. 1996. Interferon γ production by natural killer (NK) cells and $NK1.1^+$ T cells upon NKR-P1 cross-linking. *J. Exp. Med. 183: 2391.*
42. Denis, M. 1992. Mouse hypersensitivity pneumonitis: depletion of NK cells abrogates the spontaneous regression phase and leads to massive fibrosis. *Exp. Lung Res. 18:761.*
43. Takeda, K., and G. Dennert. 1993. The development of autoimmunity in C57BL/6 lpr mice correlates with the disappearance of natural killer type 1-positive cells: evidence for their suppressive action on bone marrow stem cell proliferation, B cell immunoglobulin secretion, and autoimmune symptoms. *J. Exp. Med. 177:155.*
44. Zhang, B-N., T. Yamamura, T. Kondo, M. Fujiwara, and T. Tabira. 1997. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J. Exp. Med. 186:1677.*
45. Warren, H. S. 1996. NK cell proliferation and inflammation. *Immunol. Cell Biol. 74:473.*
46. Powrie, F., J. Carlino, M. W. Leach, S. Mauze, and R. L. Coffman. 1996. A critical role for transforming growth factor-β but not interleukin 4 in the suppression of T helper type 1-mediated colitis by $CD45RB^{low}$ $CD4^+$ T cells. *J. Exp. Med. 183:2669.*
47. Sarin, A., C. M. Conan, and P. A. Henkart. 1995. Cytotoxic effect of TNF and lymphotoxin on T lymphoblasts. *J. Immunol. 155:3716.*
48. Kägi, D., B. Ledermann, K. Bürki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature 369:31.*
49. Arase, H., N. Arase, and T. Saito. 1995. Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J. Exp. Med. 181:1235.*
50. Chambers, B. J., M. Salcedo, and H-G. Ljunggren. 1996. Triggering of natural killer cells by the costimulatory molecule CD80 (B7–1). *Immunity 5:311.*
51. Geldhof, A. B., M. Moser, L. Lespagnard, K. Thielemans, and P. D. Baetselier. 1998. Interleukin-12-activated natural killer cells recognize B7 costimulatory molecules on tumor cells and autologous dendritic cells. *Blood 91:196.*
52. van Tol, E. A. F., H. W. Verspaget, A. S. Peña, C. V. E. Kraemer, and C. B. H. W. Lamer. 1992. The CD56 adhesion molecule is the major determinant for detecting non-major histocompatibility complex-restricted cytotoxic mononuclear cells from the intestinal lamina propria. *Eur. J. Immunol. 22:23.*